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In situ hybridization was used to study the ionotropic subtypes of the glutamate receptor in the rat hypothalamus, particularly in the suprachiasmatic nucleus. Widespread expression of AMPA. kainate, and NMDA receptor RNA was found in the hypothalamus. GluR1 and GluR2 were among the most strongly expressed of the non-NMDA ionotropic receptors. Other AMPA-preferring receptors, GluR3 and -R4, were also found, but to lesser extent. Scattered cells expressed the kainate-preferring receptors Glu-R5 and -R6. Little GluR7 was found in the hypothalamus. The N-methyl d-aspartate receptor, NMDAR1, was detected throughout the hypothalamus. In many regions of the hypothalamus, only scattered cells showed detectable expression of the glutamate receptor mRNA as detected by autoradiographic silver grains over neurons; unlabeled cells were mixed among labeled cells.

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March 8, 1994

Dear Ms.McKee:

Ms.Judith Dye of Yale Medical School Grants and Contracts Office indicated that my technical report may not have been received, or may not have been forwarded to your office. You and I spoke this morning, and I indicated that I thought that this had been sent in. You asked for an additional copy to be sent to your attention.

I enclose a copy of my recent progress report. This was published in J.Biol.Rhythms under the auspices of the AFOSR under the direction of Dr.G.Haddad, and described in detail much of the technical progress we have made in the 1992/1993 fiscal year. A the end of the article are listed six papers that have been published or are in press (1992/1993). These I have put a checkmark next to. I also enclose a more lengthy description of some additional experiments, and a list of the additional publications in the 1992/1993 fiscal year. These items should function as the techinical report in the event it was not received.

No inventions or patent applications were filed during this period.

I am currently doing sabbatical work at Stanford, but will return to Yale in a couple of months. If I can provide more information, or if anything else is needed please don't hestitate to call or write to me at Stanford. Thankyou for calling this matter to our attention.

Sincerely,

Anthony N. van den Pol, PhD
Professor, Yale Neurosurgery

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Dept.Biological Sciences, Stanford University, Stanford, CA. 94305
Stanford phone: 415 725-8175

cc: Ms.Judith Dye

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Progress Report 1992/1993 AFOSR.

In situ hybridization was used to study the ionotropic subtypes of the glutamate receptor in the rat hypothalamus, particularly in the suprachiasmatic nucleus. Widespread expression of AMPA, kainate, and NMDA receptor RNA was found in the hypothalamus. GluR1 and GluR2 were among the most strongly expressed of the non-NMDA ionotropic receptors. Other AMPA-preferring receptors, GluR3 and -R4, were also found, but to a lesser extent. Scattered cells expressed the kainate-preferring receptors Glu-R5 and -R6. Little GluR7 was found in the hypothalamus. The N-methyl d-aspartate receptor, NMDAR1, was detected throughout the hypothalamus. In many regions of the hypothalamus, only scattered cells showed detectable expression of the glutamate receptor mRNA as detected by autoradiographic silver grains over neurons; unlabeled cells were mixed among labeled cells. Every region of the hypothalamus including the suprachiasmatic nucleus had several different glutamate receptors. The expression of many different types of ionotropic glutamate receptors throughout the hypothalamus suggests that multiple modes of ion channel regulation by glutamate probably operate here, and provides further support for the importance of the excitatory transmitter glutamate in hypothalamic regulation.

Glutamate and regulation of circadian rhythms. The SCN functions as the circadian clock in the mammalian brain. The retinosuprachiasmatic pathway is the primary mode of sensory information into the hypothalamus that allows the endogenous circadian clock to be phase shifted in response to photic stimulation. A growing body of evidence supports the conclusion that glutamate is the primary transmitter of this retinal projection to the hypothalamus. This is based on observations that: glutamate is released upon stimulation of the optic nerves (Liou et al,1986), the response to retinal or optic nerve stimulation is excitatory (Shibata et al,'84;'86; Groos and Meijer,'85; Miller et al,'87), glutamate immunoreactivity is found in presynaptic axons in the optic nerve and SCN (van den Pol et al,'92), and blocking glutamate receptors eliminates the response of SCN cells to retinal or optic nerve stimulation (Cahill and Menaker,'89; Kim and Dudek,'92). In our in situ hybridization studies we find that a number of glutamate receptors are expressed in the SCN, including subtypes from the AMPA, kainate, and the NMDA families, suggesting that at the level of the receptor/channel in the membrane, a number of different physiological responses to light would be expected, depending on the expression of the different receptors in individual cells.

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Glutamate and GABA Presence and Action in the Suprachiasmatic Nucleus

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Abstract Glutamate and γ-aminobutyrate (GABA) are two neurotransmitters that appear to play an important role in the hypothalamic suprachiasmatic nucleus (SCN) and in the adjacent areas of the medial hypothalamus. Converging evidence based on ultrastructural immunocytochemistry, molecular biology, calcium imaging, and electrophysiology suggests not only that GABA and glutamate are used for inhibitory and excitatory activity, respectively, in this region of the rat brain, but that these two fast-acting amino acids may account for the majority of neurotransmission there.

Key words calcium, NMDA, glutamate receptor, astrocyte, retinohypothalamic, peptide colocalization

ULTRASTRUCTURAL IMMUNOCYTOCHEMISTRY

Studies using immunocytochemistry and *in situ* hybridization indicate that γ-aminobutyrate (GABA), an inhibitory transmitter, and its synthesizing enzyme, glutamate decarboxylase, appear to exist in the majority of suprachiasmatic nucleus (SCN) neurons (Tappaz et al., 1983; Card and Moore, 1984; van den Pol and Tsujimoto, 1985; Okamura et al., 1989; Moore and Speh, 1993). Functionally, administration of GABA agonists has been reported to phase-shift the circadian rhythm (Smith et al., 1989). That GABA is physiologically an important transmitter in the SCN has been demonstrated with GABA antagonists; virtually all the inhibitory postsynaptic potentials are absent after blockade with the GABA antagonist bicuculline (Kim and Dudek, 1992). On the basis of ultrastructural postembedding immunogold staining, GABA immunoreactivity was found in about half of all presynaptic endings in the SCN, as elsewhere in the medial hypothalamus. Similar results with pre-embedding ultrastructural cytochemistry were found with antiserum against glutamate decarboxylase (Decavel and van den Pol, 1990).

Glutamate appears to be the primary active transmitter in the retinal projection to the SCN, and therefore may play an important role in the phase shift of the circadian clock in response to environmental light cues. Physiological studies of the retinal input to the SCN have indicated that glutamate may be released from optic nerves (Liou et al., 1986), and that physiological responses of SCN cells to optic nerve or retinal stimulation can be blocked by glutamate receptor blockers (Shibata et al., 1986; Cahill and Menaker, 1987, 1989; Kim and Dudek, 1991).

To address the question of whether glutamate is present in SCN axons, antisera were raised against glutamate, and antiserum specificity was confirmed with enzyme-linked immunosorbent assay (ELISA), immunodot blot, Sepharose beads conjugated with amino acids, and

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Western blots. Axons were found in the SCN that demonstrated high levels of glutamate immunoreactivity, as revealed by high densities of immunogold particles in some axons (van den Pol, 1991a). Some axons in the optic chiasm ventral to the SCN were also immunoreactive for glutamate. Immunogold ultrastructural cytochemistry showed that glutamate-immunoreactive axons in synaptic contact with SCN dendrites were surrounded by astrocytes, effectively isolating the synaptic complex from other neurons, and reducing diffusion of neurotransmitter from the synaptic complex.

To study the ultrastructure of axons with high levels of immunoreactivity for GABA and glutamate, we performed postembedding immunogold staining. Consistent with earlier suggestions that synaptic specializations associated with excitatory axons were different from those associated with inhibitory axons, synaptic specializations of GABA-immunoreactive presynaptic boutons were generally symmetrical, whereas synapses associated with boutons containing high levels of glutamate immunoreactivity were generally asymmetrical. A small percentage of presynaptic boutons did not conform to the expected morphological characteristics for a particular transmitter. On the basis of reconstruction of serial ultrathin sections through many individual boutons, GABA-immunoreactive (Decavel and van den Pol, 1990, 1992) and glutamate-immunoreactive (van den Pol, 1991a) presynaptic axonal endings consistently had both clear and dense-core vesicles, suggesting colocalization of peptides with these amino acids throughout the SCN and surrounding hypothalamus.

A schematic diagram of some possible glutamate and GABA efferents and afferents is depicted in Figure 1.

IN SITU HYBRIDIZATION

To complement the localization of glutamate immunoreactivity in the SCN, we investigated the existence of glutamate receptors with *in situ* hybridization, patch clamp recording, and calcium imaging. Glutamate receptor gene expression was studied with *in situ* hybridization,

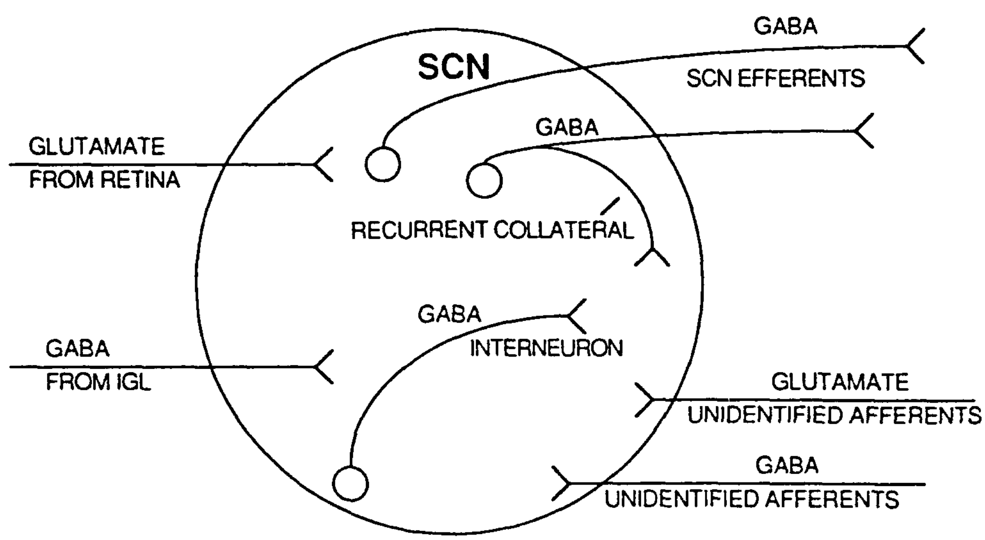

FIGURE 1. Based on anatomical, cytochemical, and electrophysiological data (see also Dudek et al., this issue), some possible efferents and afferents of the SCN that utilize GABA or glutamate are shown. Other transmitters and colocalized peptides are also found in the SCN, but are not shown in this diagram. Supporting citations are found in the text. IGL, intergeniculate leaflet.

using [^{35}S]labeled complementary RNA coding for different subunits of the glutamate receptor gene (Hollmann et al., 1989; Bettler et al., 1990; Boulter et al., 1990; Keinanen et al., 1990). GluR1, -R2, -R4, -R5, and -R6, representing subtypes of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate families of glutamate receptors, were weakly expressed in the SCN, with some differences in grain density in different regions of the nucleus (van den Pol et al., 1992b). The *N*-methyl-D-aspartate (NMDA) receptor NMDAR1 was also expressed by SCN neurons, as detected by *in situ* hybridization (van den Pol et al., 1993a). There was also a low level of expression of the metabotropic glutamate receptor mGluR1a (van den Pol et al., 1993b).

PATCH CLAMP RECORDING

Whole-cell patch clamp recordings from monolayer cultures of medial hypothalamus that included the SCN, but were not restricted to it, revealed the widespread presence of both functional NMDA and non-NMDA types of glutamate receptors. In 90% of the voltage-clamped neurons tested, an NMDA-induced inward current was detected. Non-NMDA receptors accounted for most of the depolarization in glutamate-treated current-clamped neurons (van den Pol and Trombley, 1993). A similar predominance of non-NMDA receptors was found in slices of adult rat neuroendocrine hypothalamus in the arcuate and paraventricular nuclei studied with sharp electrodes (van den Pol et al., 1990). All medial hypothalamic neurons tested *in vitro* with patch clamp recording had functional glutamate receptors.

Excitatory postsynaptic potentials (EPSPs) were recorded from single neurons and simultaneously from pairs of cultured current-clamped medial hypothalamic neurons. Blocking NMDA and non-NMDA glutamate receptors with *d,l*-2-amino-phosphonovaleric acid (100 μM AP5) and cyano-2,3-dihydroxy-7-nitroquinoxaline (3 μM CNQX) reduced the EPSPs in all neurons tested, suggesting not only that these cells maintain glutamate receptors, but that medial hypothalamic cells release glutamate as an excitatory neurotransmitter. We found that almost all of the spontaneous or evoked excitatory activity in medial hypothalamic cultures could be blocked by glutamate receptor antagonists, suggesting the widespread release of transmitter glutamate by hypothalamic neurons. Given the incidence of GABA in most SCN perikarya, it appears unlikely that many SCN cells themselves would release glutamate.

CALCIUM DIGITAL IMAGING

NEURONS

Ca^{2+} imaging dyes fluo-3 and fura-2 were used with digital video microscopy to study Ca^{2+} fluxes in response to glutamate and GABA. SCN neurons in dispersed cultures showed Ca^{2+} rises in response to the glutamate agonists kainate, quisqualate, and NMDA, and to glutamate. Neurons sometimes showed spontaneous ultradian oscillations; most of these were irregular in period length. Some neurons showed regular oscillations with periods in the range of 10–30 sec; the amplitude of the Ca^{2+} peaks in these oscillating neurons could be increased with addition of glutamate, or could be blocked by the addition of GABA.

In cultures of medial hypothalamic neurons, calcium levels, elevated as a result of the putative release of glutamate from other hypothalamic neurons, were decreased by the addition of the glutamate blockers AP5 and CNQX to the perfusion buffer (van den Pol and

Trombley, 1993). These experiments provide further support for the idea that hypothalamic neurons release glutamate as an excitatory transmitter.

GLIA

SCN astroglia routinely showed regular ultradian oscillations with periods in the range of 10–20 sec in response to glutamate, as well as to serotonin and extracellular adenosine triphosphate. Long-distance astrocyte signaling, probably via astrocytic gap junctions, could be elicited with glutamate application to dispersed cells or organotypic slices of SCN in the microscope perfusion chamber. Confocal scanning laser-microscopic analysis of SCN roller tube explants showed that some astroglia had very regular endogenous Ca^{2+} ultradian oscillations with relatively long periods (1–2 min) (van den Pol et al., 1992a). Cytochemically, SCN astrocytes are different from those in the surrouding hypothalamus, in that the general level of glial fibrillary acidic protein is greater within the SCN than outside it (Morin et al., 1989), despite the fact that the relative number of astrocytes in the SCN is small (van den Pol et al., 1992a). Possible mechanisms by which astrocytes might influence SCN neuronal activity include regulation of extracellular potassium or calcium, or arachidonic acid modulation, as described in detail elsewhere (van den Pol et al., 1992a; van den Pol and Dudek, 1993).

CONCLUSION

Together, these data support a prominent role for the amino acid transmitters glutamate and GABA in SCN function. Many other neuroactive substances can also be found in the SCN, as a number of investigators have demonstrated (Sofroniew and Weindl, 1978; Card et al., 1981; Card and Moore, 1984; van den Pol and Tsujimoto, 1985; see van den Pol, 1991b, and van den Pol and Dudek, 1993, for a review), and neuropeptides administered to the SCN can cause phase shifts in the circadian rhythms (Albers et al., 1991). A major role for neuroactive peptides localized in the SCN may be to modulate the activity of GABA-ergic and glutamatergic postsynaptic responses, similar to the modulatory role of dopamine in the facilitation of glutamate responses in the retina (Knapp and Dowling, 1987).

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